HYDROGEN-ION STUDIES

III. HYDROGEN-ION CHANGES IN THE AGGLUTINATION OF BACTERIA BY IMMUNE SERUM *

EDWIN F. HIRSCH

From the Pathological Laboratory of St. Luke's Hospital, Chicago

The agglutination of a suspension of bacteria by homologous immune serum in the presence of a salt is one of the best known of the immune reactions. A highly potent immune serum in proper dilutions agglutinates the suspended bacteria into masses which collect at the bottom and sides of the test tube, and the liquid in which the bacteria are suspended becomes clear. Until nonmotile bacteria were observed to agglutinate with immune serum, agglutination was thought to result from some change of the flagella. Then some alteration of the ectoplasm by the immune serum whereby the bacteria became adhesive was considered the cause of agglutination. That bacteria are purely passive however, was learned by observing agglutination with bacteria killed by heat or by various chemicals.

Bordet [1] made the important observation that a salt such as sodium chlorid is necessary for the agglutination of bacteria by immune serum. He also observed that the agglutinins combined with the bacteria in the absence of salt and that the compound so formed was precipitated by the addition of small quantities of salt.

Bechhold [2] and others have shown that bacteria in salt solution carry a negative electrical charge. After having been acted on by agglutinin, they precipitate between the electrodes. In all respects, Wells [3] says, the behavior of bacteria and agglutinins resembles the behavior of colloidal suspensions which form an electrically amphoteric colloidal suspension so that the ions of the electrolytes, or the electrical currents by discharging them unequally, cause precipitation. According to Stieglitz,[4]

Received for publication Feb. 23, 1922.

* Aided by the Winfield Peck Memorial Fund.

[1] Ann. de l'Inst. Pasteur, 1899, 13, p. 225.

[2] Ztschr. f. physik. Chem., 1904, 48, p. 385. Colloids in Biology and Medicine, Trans. by J. G. M. Bullowa, 1919.

[3] Chemical Pathology, 1920.

[4] Qualitative Chemical Analysis I, 1913.

colloids which in a solution carry a negative electrical charge are readily precipitated by the action of positive ions, positively charged colloids by the action of negative ions (Hardy's rule). The precipitated colloid carries with it a part of the precipitating ion (absorption), and the weights of the ions carried down by a given quantity of a given colloid are proportional to the equivalent weights of the ions. The precipitation thus appears to be intimately associated with the neutralization of the charge on the colloid. In accordance with this conclusion, it has been found that a colloid may be precipitated by a colloid carrying an opposite electrical charge.

Colloidal protein solutions now are generally considered to be solutions of amphoteric electrolytes, and the colloidal particles carry a negative electrical charge in an alkaline medium and a positive charge in an acid medium. At a hydrogen-ion concentration specific for the protein there exists no difference in the electrical potential between the particles and the medium, so that the particles appear to be without an electrical charge. This is known as the iso-electric point. Loeb [5] has demonstrated that this marks the turning point of the chemical change which determines the nature of ionization, and the ionization determines the electrical charge. When ionized as an acid and combined with a cation on the alkaline side of the iso-electric point, the protein particle behaves as an anion and carries a negative electrical charge; when ionized as a base and combined with an anion at reactions more acid than the iso-electric point, the protein carries a positive charge.

Coulter [6] determined P_H 4.6 as the iso-electric point for red blood cells (sheep). At hydrogen-ion concentrations less than P_H 4.6, the charge carried by the red blood cells is negative and increases in amount with the alkalinity; at concentrations greater than P_H 4.6, the electrical charge is positive and increases with the acidity. He observed further, that the chlorine ion in the presence of sodium chloride, combines with both normal and sensitized cells in much larger amounts on the acid side of P_H 4.7 than on the alkaline side. Adding sodium hydroxide actually liberates chlorine from the cells between the reactions of P_H 5.7 and P_H 6.2. Similar observations have been made by Loeb,[5] who found that gelatin on the alkaline side of its iso-electric point combines only with a cation, and on the acid side only with an anion. These observations suggest that similar ionization changes occur with bacterial pro-

[5] Jour. Gen. Physiol., 1919, 1, pp. 39, 237, 363, 483, 559.
[6] Ibid., 1920, 3, p. 309.

teins, and that in the presence of an electrolyte such as sodium chloride, a salt of the bacterial protein and the cation of the electrolyte is formed on the alkaline side of its iso-electric point.

In another study Coulter [7] observed the equilibrium between hemolytic sensitizer and red blood cells in relation to the hydrogen-ion concentration. He says that the amphoteric electrolytes with which the immune bodies must be classed on the basis of their behavior in the electrical field owe their electrical charge to ionization, and the combination of sensitizer and cells is related intimately with the ionization of the immune body. In a salt-free medium, he finds the proportion of the total amount of hemolytic sensitizer present and combined with the homologous cells is almost 100% at P_H 5.3. On the alkaline side of this reaction, the proportion diminishes with increasing alkalinity to about 5% at P_H 10. On the acid side, the same decrease occurs, but somewhat less rapidly. The presence of sodium chloride greatly increases the proportion of sensitizer combined with cells at all reactions except those about P_H 5.3. At this point the combination of sensitizer with cells is independent of the electrolyte.

Loeb [8] regards the difference in the hydrogen-ion concentration between the micellae of protein and the surrounding solution as the only cause of the electrical charges of the micellae of protein or of their models. The electrical charges of powdered gelatin suspended in an aqueous solution are determined by the fact that acid is forced into the water solution when the particles consist of gelatin chloride, and that alkali is forced into the water when they consist of sodium gelatinate. The P_H inside minus the P_H outside the particles is positive as long as the P_H of the gelatin is on the acid side of its iso-electric point, while it is negative when the gelatin is on the alkaline side of its iso-electric point. Addition of salt to solutions containing powdered gelatin chloride diminishes the potential difference between the particles and the surrounding liquid, and this is due to a diminution of the value of P_H inside minus P_H outside.

Since many chemical reactions are accompanied by changes in the hydrogen-ion concentration of the medium in which they occur, and as such changes are significant in understanding these chemical reactions, it seems possible that a study of the hydrogen-ion concentration of the medium in which agglutination of bacteria by immune serum

[7] Ibid., 1920, 3, p. 513.
[8] Ibid., 1922, 4, p. 351.

takes place may add new facts which will aid in understanding better the nature of this and similar reactions.

The immune serums were prepared in rabbits. The bacterial suspensions were made with normal salt solution, and the growth of bacteria obtained from 24-hour plain agar slant cultures. Such suspensions were diluted to a medium density and then heated to 56 C. for half an hour to kill the bacteria. To 1 c c quantities of serum diluted with normal salt solution beginning with 1:25 and continuing to 1:6,400 were added 1 c c quantities of the suspension. This system of preparing the dilutions was followed throughout. The mixtures were incubated with their controls (similar dilutions of nonimmune serum) at 37 C. overnight, and the hydrogen-ion concentration of each was determined the next morning. These estimations were made at a constant temperature (25 C.) by the gas-chain method.

The chart illustrates graphically the results of such an experiment and its control with normal serum.

Results which in graphs make curves of similar contour have been obtained with suspensions of typhoid bacilli, colon bacilli, dysentery bacilli, and paratyphoid bacilli, and their homologous immune serums.

COMMENT

The interpretation of the changes in the reaction of the medium in which agglutination occurs involves consideration of certain ionization principles. When sodium chloride is added to water, ionization occurs as follows:

$$\begin{array}{ccc} NaCl & Na^{+} & Cl^{-} \\ HOH & OH^{-} & H^{+} \\ & \downarrow\uparrow & \downarrow\uparrow \\ & NaOH & HCl \end{array}$$

Since the dissociation constants of sodium hydroxide and hydrogen chloride are so nearly equal, such a solution contains an equal concentration of hydrogen and hydroxyl ions and reacts neutral. The addition of bacteria (living or killed) renders the reaction alkaline, as for example the medium changes from P_H 7 to P_H 8.25, a change not due to substances dissolved from the plain agar on which the bacteria were cultivated. The bacteria themselves carry negative electrical charges. Similar changes in reaction are observed when the salt of a strong base and a weak acid is ionized in water. This behavior of bacteria in salt solution suggests that the bacterial protein combines

with the Na ion to form a salt, or that this salt already exists when the bacteria are added, and that it then dissociates. The latter seems quite likely because practically all mediums contain salt.

Bacteria in salt solution must be considered to be in two phases, a dissolved portion [9] and an undissolved portion. The latter is concerned especially with the physical changes of agglutination. The dissolved portion ionizing as the salt of a strong base and a weak acid probably causes the alkaline reaction. Letting NaBa represent the dissolved protein salt, its ionization would occur as follows:

Na Ba	Na^{+}	Ba^{-}
H OH	OH^{-}	H^{+}
	$\downarrow\uparrow$	$\downarrow\uparrow$
	NaOH	HBa

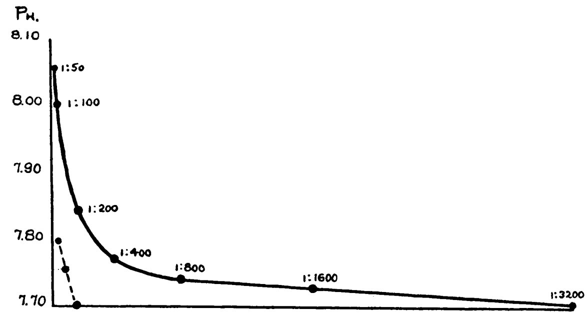

Chart 1.—Graphs representing the reaction of dilutions of serum and colon bacillus suspension; the solid line represents those with immune serum, the interrupted line those with nonimmune serum. Agglutination was complete in dilutions of 1: 50 and 1: 100 and decreased to slight in dilution of 1: 3,200. No agglutination with control serum.

The dissociation of sodium hydroxide is so nearly complete that very little sodium hydroxide exists as such in the liquid. The dissociation of many weak organic acids, however, is much less, and in a medium in which it is establishing its equilibrium, hydrogen-ions are used up, more water ionizes to satisfy its dissociation constant, and the formation of undissociated weak acid continues until its dissociation constant and the dissociation constant of water are satisfied. The hydrogen ion is therefore used up to a much greater degree than is the hydroxide ion. The latter accumulates and the solution becomes alkaline.

[9] See discussion of soluble bacterial substances by Martin Ficker, Kolle and Wassermann, Handbuch der path. Mikroorganismen, 1913, 2.

The mixtures of bacteria and homologous immune serum in the agglutination experiments mentioned are as follows:

Bacterial suspension.	1.0 c c	1.0 c c	1.0 c c
Salt solution.........	0.96 c c	0.98 c c	0.99 c c, etc.
Immune serum	0.04 c c	0.02 c c	0.01 c c

As the amount of immune serum decreases in each tube, the extreme limit of reaction on one end of the series of dilutions (greatest) is that of the bacterial suspension diluted with an equal volume of salt solution, and at the other end of the series it is this reaction modified by the substitution of 1/50th volume salt solution by immune serum. Similar relations exist for mixtures of nonimmune serum and bacterial suspension.

The curve representing the hydrogen-ion concentration of the dilutions of the homologous immune serum and bacterial suspension demonstrates an increase in the alkalinity of the medium where this occurs, while that representing those for the nonimmune serum is a straight line, in all respects like a graph of hydrogen-ion concentrations obtained by similar dilutions of two chemically inert solutions with different reactions.

The negative electrical charges carried by bacteria probably are acquired in much the same way as are similar electrical charges by the colloidal particles of a metal such as platinum. Such colloidal particles react with water whereby an incomplete chemical combination with the liquid occurs. $_{n}Pt.^{+} H.OH = (Pt_{n} H^{+})^{+} OH^{-}$. This platinum-hydrogen aggregate dissociates slightly and gives rise to a negative charge on the metal.[10]

Bordet's[1] observation that the presence of a salt is necessary for agglutination may be taken to mean that in distilled water the bacterial protein is not ionized to an appreciable extent. In order to react with immune serum and agglutinate, ionization is necessary and occurs when the bacterial protein is combined with a base to form a salt. The distribution of electrical charges is represented then by $Na^{+} Ba^{-}$.

The agglutination of bacteria is associated with the neutralization of their electrical charges by the immune substance. **It is fair to believe** that the immune body carries a positive electrical charge, that it possesses basic properties, and that it ionizes according to the formula $\frac{\text{Immune substance x OH}}{\text{Immune substance OH}^{-}} = K_{\text{I. S.}}$ The dissociation constant $K_{\text{I.S.}}$ is satisfied in the serum, so that adding serum to the salt solution-

[10] Burton, E. F.: The Physical Properties of Colloidal solutions, 1916.

bacterial suspension mixture alters the reaction only in so far as its dissociated products are able. The neutralization of the negative charge of the bacteria by the immune body liberates the cation of the bacteria (Na) and the anion of the immune body (OH). The dissociation constant of the sodium hydroxide resulting from this interaction is probably so much greater than the dissociation constant of the immune substance that more hydroxyl ions are contained in the liquid after agglutination than before, and the medium becomes more alkaline.

The contour of the graph representing this reaction strikingly resembles others representing titration and dissociation curves.[11]

The intravenous injection of a suspension of bacteria[12] has been found to lower temporarily the alkaline reserve of the blood. Within 24 hours, when recovery is prompt, the alkaline reserve of the blood returns to normal, sometimes after having reached a higher than normal value. These changes have been regarded as significant in the establishing of immunity. Since bacteria in their chemical activity resemble the salts of weak acids, the lowering of the alkaline reserve of the blood by their presence is not difficult to correlate. Nor is it unlikely that the immune substance of an acid salt is basic in reaction. In regarding the balanced P_H of the blood, it is necessary to consider the inorganic substances, carbonates (notably) and the phosphates, which fluctuate rapidly and easily to maintain the P_H equilibrium, and another group (proteins), the basic immune substances, which appear in defense against infections, and which alter by their dissociation products the reaction of the blood. The latter changes probably are very small and are readily compensated by the inorganic substances first mentioned.

SUMMARY

Bacteria suspended in normal salt solution behave chemically and electrically like the anion of the salt of a strong base and a weak acid.

When bacteria are agglutinated by homologous immune serum, the medium in which this reaction occurs increases in alkalinity.

This change in reaction is regarded to result from differences in the dissociation constants of the reacting substances and their products.

[11] Clark, W. Mansfield: The Determination of Hydrogen Ion, 1920.

[12] Hirsch, E. F.: Jour. Am. Med. Assn., 1920, 75, p. 1204; Jour. Infect. Dis., 1921, 28, p. 275.